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Original Article

Effects of cigarette smoke condensate and nicotine on growth status and cytokine expression of human gingival fibroblast on titanium plate

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Abstract: Human gingival fibroblasts (HGFs) are important for protecting and repairing periodontal tissues. Smoking is a factor that may induce failure of dental implantation. This study investigated the effect of cigarette smoke condensate (CSC) and nicotine on the attachment, growth or expression of interleukin (IL)-6, -8 in HGFs on titanium plate, to uncover the mechanism of smoking in destructing implant gingival interface. Primary cultured HGFs were identified by immunohistochemistry (IHC) staining under SP approach. SRB method was used to detect the adhesion and proliferation of HGFs under different concentrations of CSC. The spreading area and shape index changes were analyzed by a pathology imaging system. Cell adhesion structure was scanned in EM. Levels of IL-6 and IL-8 in the supernatant were detected by ELISA. HGFs showed positive expression of vimentin and negative expression of keratin. Surface adhesion, proliferation, shape index and spreading area were decreased in HGFs after treatment with higher CSC or nicotine concentrations. Such inhibitory effects and IL-6/IL-8 levels were elevated after treated by higher concentrations ($P < 0.05$) in a dose-dependent manner. Certain concentration of CSC and nicotine can inhibit the attachment and proliferation of HGFs in titanium plate, and facilitate IL-6/IL-8 synthesis and secretion, indicating that toxic substance in cigarette may exert adverse effects on implant gingival interface via modulating the expression of IL-6 and IL-8.

Keywords: Cigarette smoke condensate, nicotine, human gingival fibroblasts, cytokines

Introduction

Smoking has become a major health concern for people's health. Toxic substances such as nicotine, acrolein, and cotinine can cause a series of oral cavity issues [1]. Human gingival fibroblasts (HGFs) locate gingival mesenchymal tissues, and play an important role in maintaining the normal structure of gingival and remodeling process. Moreover, the long-term stability of dental implant is closely associated with HGFs [2]. Some studies showed that nicotine could inhibit HGFs proliferation in a dose-dependent manner. Cigarette extracts can cause abnormal structure of HGFs, cellular DNA injury and facilitate HGFs apoptosis. Tobacco can also cause breakage of double stranded DNA in oral fibroblast [3, 4]. Low concentration of cotinine and nicotine can decrease the adhesion of HGFs precursors on dental slices, whilst high dosage of cotinine and nicotine can further inhibit the adhesion or proliferation of HGFs [5, 6]. Body inflammation and wound healing all involve leukocytes. The level of IL-5 and IL-1β in serum and gingival clef fluid was different between healthy and patients with periodontitis, which is believed to be related with inflammatory factors [7, 8]. Currently various studies have been performed on the relationship between smoking and inflammation, but with inconsistent results. Some studies believed that IL-1α was up-regulated in HGFs under nicotine treatment, whilst IL-1β and IL-1α secretion were enhanced under CSC [9, 10]. Some studies believed no statistically significant differences in IL-6 or IL-8 in gingival cleft fluid between healthy individuals and patients with inflammation peripheral of implants, with lower IL-6 or IL-8 expression in HGFs under CSC effects [11, 12], possibly due to the different property of HGFs and experimental design. Therefore, the effect of ciga-

rette on HGFs related inflammation requires further study. Smoking is an important factor causing the failure of implants [13]. The study of cigarette's effects on the successful rate of implanted teeth on patients is thus of critical importance. This study investigated the effect of different concentrations of CSC and nicotine on the attachment, proliferation and cytokine IL-6/IL-8 expression of HGFs on platinum, in order to uncover the related mechanisms of cigarette on destructing implant gingival interface.

Materials and methods

Reagent and equipment

DMSO, high-glucose DMEM, trypsin, sulfa-rhodamine B, FBS were purchased from Gibco (US), Sigma (US), and Boster (China). IL-6 and IL-8 ELISA kits, anti-keratin, anti-vimentin, rabbit anti-rat actin antibody were purchased from Jiancheng Bio (China) and Zhoangshan Jingqiao Bio (China). Major equipment include: S570 scanning electron microscope (Hitachi, Japan), DM2500 fluorescent microscope (Leica, Germany), UV-2550 spectrometry (Shimazu, Japan). Titanium plate (1 mm thickness and 10 mm diameter) was purchased from Jinnaite (China). Gold-phase sandpaper was provided by Changshanjiao (China). CSC was provided from Tobacco Inc. (China) and was diluted into DMSO at 10 mg/ml.

Primary culture of HGFs

Using improved tissue cube method, healthy gingival tissues were collected from teeth removal. Tissues were rinsed in Hank's solution containing double antibiotics. Tissues cubes were removed from epithelial and cut into 1 mm^3 cubes, which were digested in trypsin and centrifuged. Supernatant was added into FBS-containing culture medium for incubation (5% CO_2, 37°C with full humidity). Cell growth condition was examined under an inverted microscope. Cells at 4^{th} to 8^{th} generation were used for further experiments.

Identification of HGFs sources

Cell motility was observed under an inverted microscope. Immunohistochemistry SP method was used with anti-keratin (1:100), anti-vimentin (1:100) antibody, followed by DAB staining to identify cell sources.

Buffing of titanium plate and assay of surface roughness

Titanium plate was buffing in different granularity of sandpaper. Reflective microscope was used to observe the surface scratching of titanium plate. Five randomly selected titanium plates were used to examine the surface roughness by Ra 2205 apparatus for calculating average values. Scanning electron microscope was used to observe the surface shape of titanium plate, which was sterilized by high pressure steam.

SRB for evaluation of the adhesion and proliferation of HGFs on titanium plate surface under gradient concentrations of CSC and nicotine

HGFs were inoculated into culture plate, which contained titanium in each well, in DMEM medium containing 10% FBS (5×10^4/ml). CSC (0, 2.5, 5 and 10 mg/ml) and nicotine (0.075, 0.15 and 0.3 mg/ml) were added for incubation for 12 h. Following 4°C culture in acetic acid monochloride for 1 h, the HGFs were washed and dried. SRB was added for staining and washing. Tris-alkaline buffer was added for 5-min vortex. Absorbance value at 515 nm was measured by a microplate reader to assess the growth condition of HGFs on titanium plate after treatment by different concentrations of CSC or nicotine for 24, 48 or 72 h.

Spreading area and shape index of HGFs on titanium surface under CSC and nicotine

Cells were incubated with different concentrations of CSC and nicotine for 12 hours. Rabbit anti-rat actin was added for overnight incubation at 4°C. Anti-rabbit IgG (FITC labelled, 1:50 dilution) was then added for incubation. After rinsing in PBS, the titanium plate was attached on the slide for storage in glycerol at 4°C. Inverted fluorescent microscope was used to observe cell spreading and shape within 72 hours. Five randomly selected fields were chosen. Cell circumstance (C) and area (A) were analyzed by pathology imaging software for calculating shape index.

Nicotine quantification in CSC

UV spectrometry was used to calculate the nicotine content in 10 mg/ml CSC based on textbook. In brief, using dilution factor F = 100, 1

Figure 1. Growth condition of HGFs and morphology of primary cultured cells. A. Minor fibroblast cell outgrowth (×100); B. Outgrowth of epithelial cells from cultured tissue cubes (×100); C. 4~7 days after culture (×200).

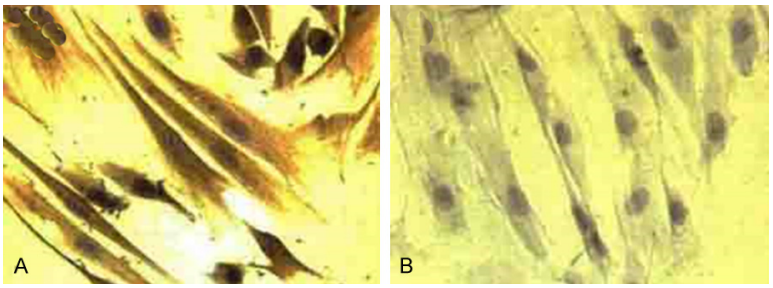

Figure 2. Identification of cell origin (×400). A. Positive staining for vimentin; B. Negative staining for keratin.

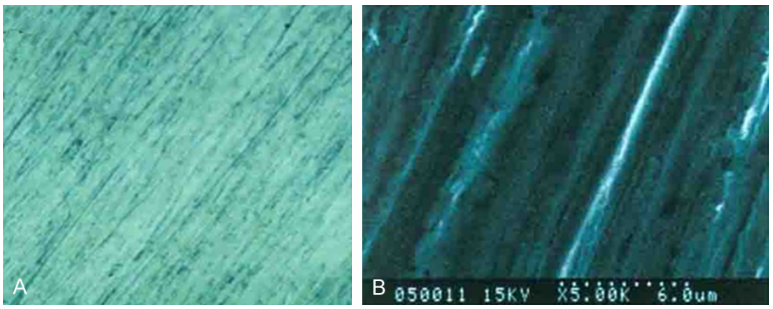

Figure 3. Roughness of titanium surface. A. Surface scratch (×200 under reflective microscope); B. Surface of titanium (×5000 under scanning electron microscope).

ml CSC medium (10 mg/ml) was diluted into 100 ml using 0.025 mol/L H_2SO_4. UV absorbance values at 236 nm, 259 nm and 282 nm were measured. At A259 = 0.282, A236 = 0.309 and A282 = 0.198, nicotine content = 0.088 g at 10 mg/ml.

Adhesion morphology and apoptosis of HGFs on titanium plate surface under CSC and nicotine

After treatment of different concentrations of CSC and nicotine for 24 hours, culture medium was removed followed by collection of titanium plate. Cell adhesion morphology was observed using methods described above.

ELISA for measuring IL-6 and IL-8 contents in supernatant

Cells were inoculated in titanium plate as abovementioned. After 24 hours, the supernatant was collected and quantified for the levels of IL-6 and IL-8 using ELISA kit following manual instructions. A microplate reader was used to measure absorbance value at 540 nm. Concentration (in pg/ml) was calculated from the standard curve.

Statistical method

SPSS19.0 software was used for analysis. Measurement data were firstly tested for normal distribution. Those fitted normal distribution were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for multiple group comparison. In-group comparison was performed using LSD test. A statistical significance was defined when $P < 0.05$.

Results

Growth condition of primary cultured HGFs

3~7 days after inoculation, tissue cube peripheral regions had out-flow cells, which showed pseudopod like or radical shape. Those newly formed fibroblast has elongated spindle on both sides with sharp protrusion. Certain orientation can be formed when cell had certain density (**Figure 1**).

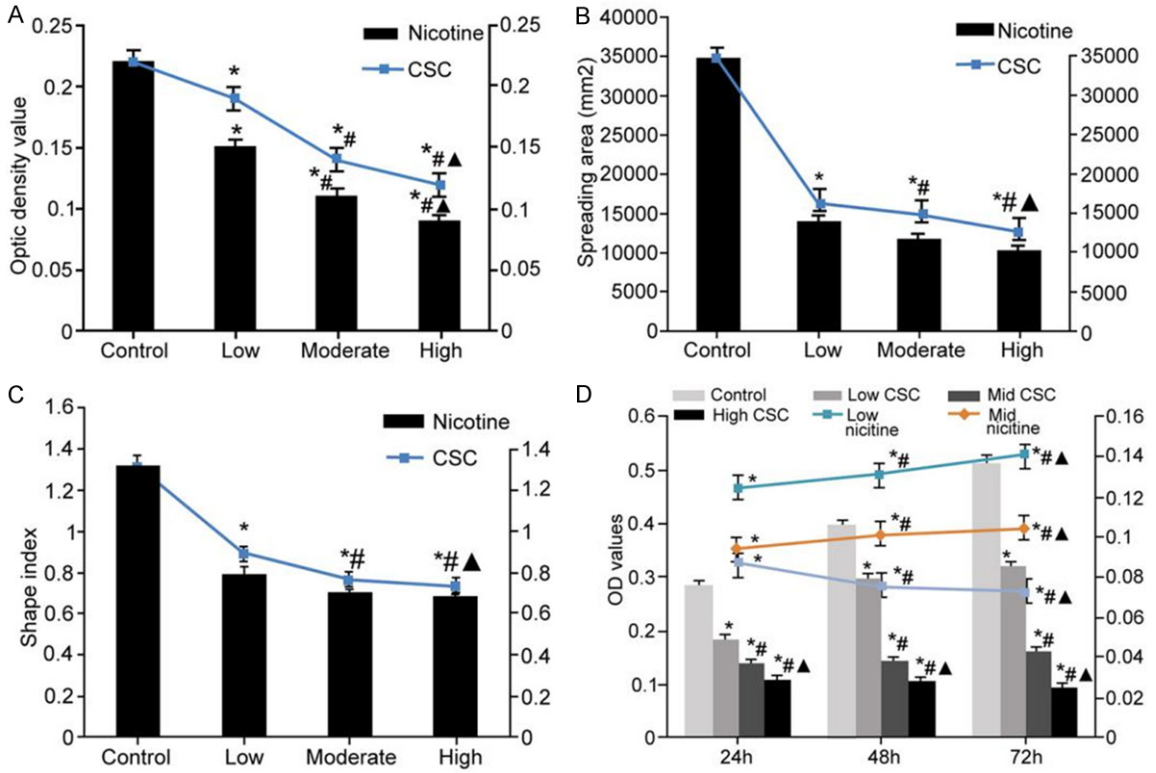

Figure 4. Effects of CSC and nicotine on surface adhesion and proliferation on titanium plate surface. Note: Negative control: CSC = 0 mg/ml; CSC low, moderate, and high dosages: 2.5, 5 and 10 mg/ml. Low, moderate and high dosage of nicotine: 0.075, 0.15 and 0.3 mg/l. *, $P < 0.05$ compared to negative group; #, $P < 0.05$ compared to lower dosage; Δ, $P < 0.05$ compared to moderate dosage.

Identification of cell origin

Cells were positive for vimentin as demonstrated by brown-yellow color of cytoplasm. IHC results confirmed the mesoderm-origin of cells (**Figure 2**).

Surface roughness of titanium plate

Under crude observation, when the titanium plate was rubbed into 1200 gauges, its surface showed reflective features. Under light microscope, the titanium surface has uniformed direction and evenly distributed scratches. Five randomly selected titanium plates were measured to have Ra values at 0.110 μm, 0.098 μm, 0.107 μm 0.096 μm and 0.102 μm, with averaged Ra values at (0.1026 ± 0.0074) μm (**Figure 3**).

HGFs adhesion and proliferation on titanium surface under CSC and nicotine

Compared with control group, the surface adhesion, proliferation, spreading area and shape index were all decreased after treatment by higher concentration of CSC or nicotine ($P < 0.05$, **Figure 4**).

Morphology structure of adhesion cells on titanium surface under different concentrations of CSC and nicotine

Under fluorescent microscope, the whole titanium surface was covered with all spindle cells showing green fluorescence. Without CSC or nicotine, cells showed swelling spindles, which were elevated after treatment by increased CSC and nicotine concentrations. Cells were gradually thinned on both sides. Under high dosage drugs, abundant dead cells occurred. Grid-like structure occurred on nicotine cell surface with dosage dependency (**Figure 5**).

Effects of CSC and nicotine on IL-6 and IL-8 expression on titanium late

Both IL-6 and IL-8 levels were elevated after treated with higher concentrations of CSC and nicotine (**Figure 6**).

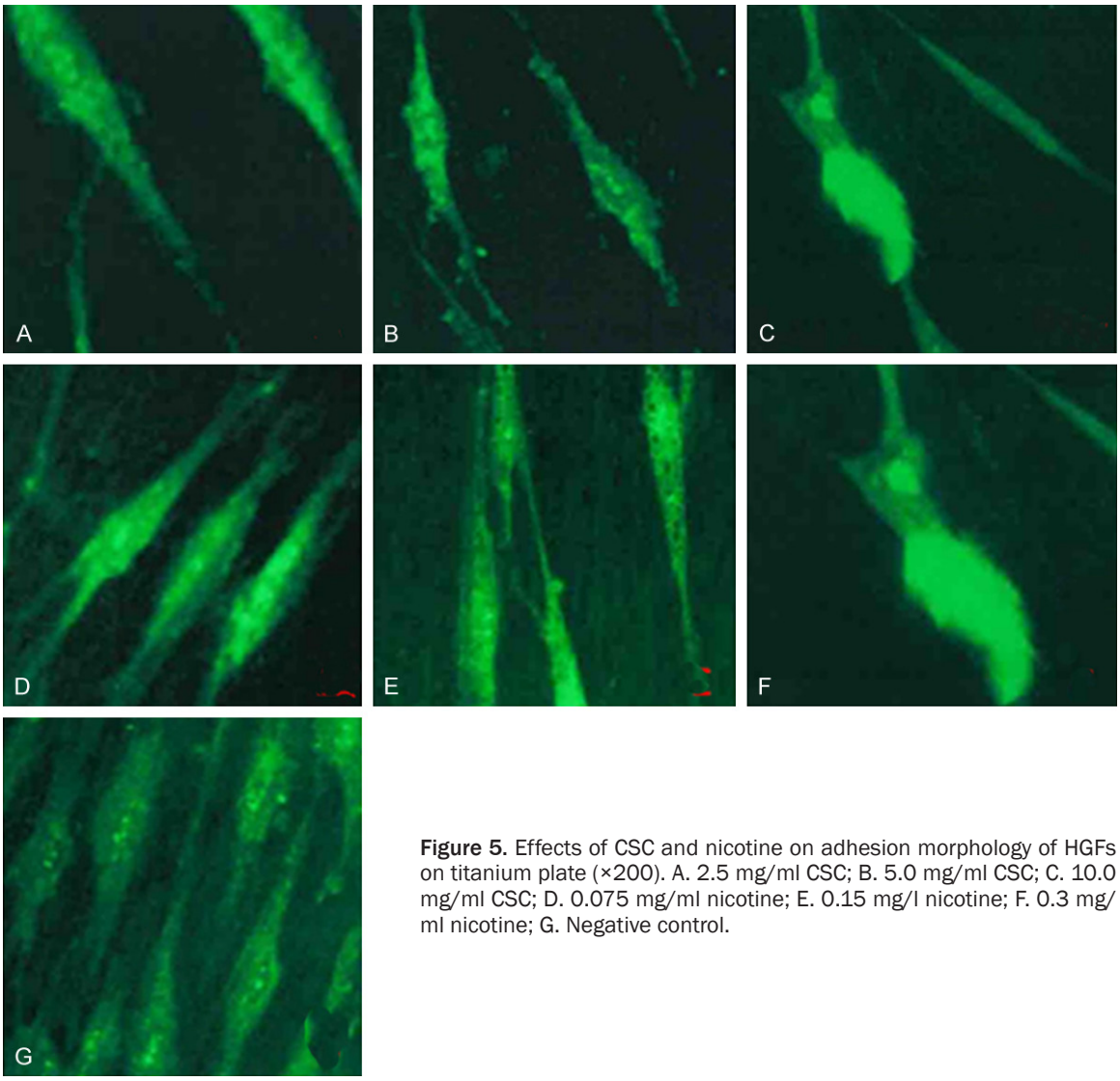

Figure 5. Effects of CSC and nicotine on adhesion morphology of HGFs on titanium plate (×200). A. 2.5 mg/ml CSC; B. 5.0 mg/ml CSC; C. 10.0 mg/ml CSC; D. 0.075 mg/ml nicotine; E. 0.15 mg/l nicotine; F. 0.3 mg/ml nicotine; G. Negative control.

Discussion

During formation and regeneration of gingival interface of dental implants, HGFs play an important role. In early phase of gingival interface healing, HGFs secrete silk-like substances covering the surface of dental implants, whose trauma was recovered by adhesions. HGFs involve in repair and reconstruction of dental implants to finish sleeve closure [14, 15]. Therefore, HGFs and their effective adhesion/proliferation around dental implants are critical for inflammatory repairmen. The binding status between implants and tissues, and functional status were affected by the surface morphology. This study utilized the mechanical rubbing to process titanium plate, whose surface shape was regular after handy rubbing, reflecting smooth structure of implant neck. The quantification of roughness on platinum surface revealed it as suitable for cell attachment.

Attachment and stretching of HGFs on material surface is key for the formation of sleeve closure of implants [16, 17]. In this study, HGFs had gradually decreased adhesion, proliferation, shape index and spreading area under treatment of elevated concentrations of CSC and nicotine, indicating that high concentration of CSC and nicotine might directly cause the injury of gingival fibroblasts, lowering the cell adhesion potency. Under low concentration, it is probably due to the change of surface electron distribution. This study also measured the nicotine level in CSC by spectrometry. In 10

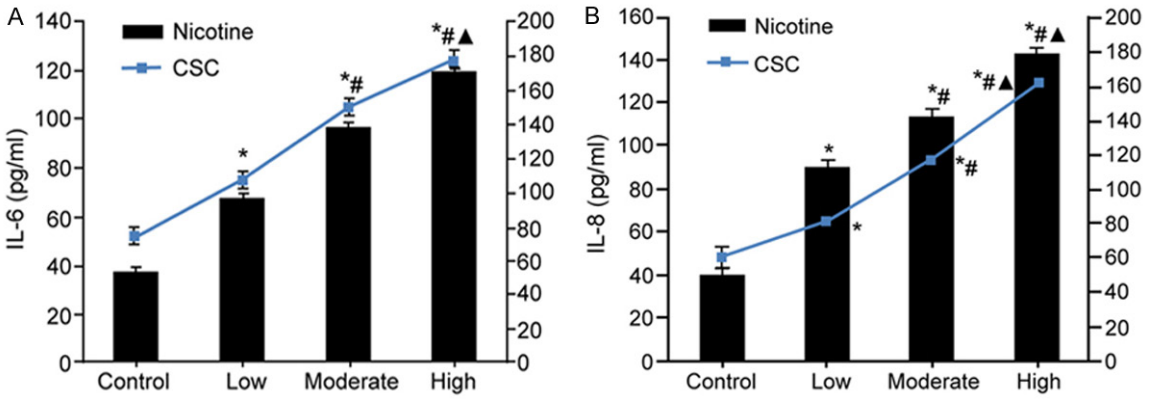

Figure 6. Effects of different concentrations of CSC and nicotine on IL-6 and IL-8 expression of HGFs on surface of titanium plate.

mg/ml CSC, nicotine concentration was 0.088 g, with lower spreading area and adhesion potency than 0.075 mg/ml nicotine groups. These results suggested that certain substances other than nicotine in CSC might participate in inhibiting spreading of gingival on titanium plate surface, such as acetaldehyde or tobacco tar. All of those factors can inhibit HGFs adhesion, destruct HGFs skeleton system, affect the spreading on surface [18, 19].

Tight binding between gingival and implants forms transform precursor. Tobacco directly affects HGFs growth and function, thus suppressing HGFs, probably due to the lower HGFs adhesion or migration which were induced by toxic components in tobacco, leading to inflammation, oxidative injury, and facilitation of extracellular matrix degradation [20-22]. Results of this study showed elevated IL-6/-8 expression in HGFs after treated with higher concentration, plus dose-dependent inhibitor effects, suggesting that certain concentration of CSC or nicotine could inhibit the proliferation or attachment of HGFs on titanium plate surface, where it facilitated IL-6 and IL-8 secretion. Therefore, toxic substances in cigarette may exert adverse effects on implant gingival interface via regulating the expression of IL-6 and IL-8. Effective measures are needed in clinics for improving the outcomes of implants in patients with smoking. This study found that under fluorescent microscope, those titanium plates without CSC or nicotine treatment were covered with spindle cells, whose level was elevated after treated with CSC and nicotine concentrations. Cells were thinned at both sides. Under high concentration, abundant cell death occurred, with mesh like structure occurrence on HGFs cell surface in a dose-dependent manner. In experiment, we used myosin-fluorescence to find the possible correlation between changing mesh structure and destruction of cytoskeleton by nicotine. Focal rupture of cytoskeleton and lower myosin density all lead to the changes of mesh structure by peripheral contraction of ruptured skeletons.

Conclusion

Certain concentration of CSC and nicotine effectively inhibit the attachment and proliferation on titanium plate surface, facilitating the synthesis and secretion of IL-6 and IL-8, indicating that toxic substances in tobacco may exert adverse effects on gingival interface, which might be via modulating the expression of IL-6 and IL-8.

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Disclosure of conflict of interest

None.

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